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Calcitonin mRNA polymorphism: Peptide switching associated with alternative RNA splicing events

(RNA processing regulation/hormone biosynthesis/functional domains/gene organization/neuroendocrine system)

MICHAEL G. ROSENFELD*, CHIJEN R. LIN*, SUSAN G. AMARA*, LANA STOLARSKY*, BERNARD A. ROOS†, ESTELITA S. ONG‡, AND RONALD M. EVANS‡

*Division of Endocrinology, School of Medicine, University of California at San Diego, La Jolla, California 92093; †Endocrinology and Mineral Metabolism, Veterans Administration Medical Center and School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106; and ‡Tumor Virology Laboratory, The Salk Institute, San Diego, California 92138

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ABSTRACT **Evidence is presented which supports a model proposing that differential RNA splicing events may be used in expression of genes of the endocrine system to generate alternative polypeptide hormones. We previously reported that variation in the expression of the gene encoding the small polypeptide hormone calcitonin is associated with the production of a new calcitonin-like or pseudo-calcitonin (ψCal) mRNA. A plasmid containing ψCal cDNA sequences has been constructed, and calcitonin genomic clones have been isolated. Hybridization analysis reveals that calcitonin and ψCal sequences are chromosomally linked and are present in the same nuclear RNA transcripts. Both calcitonin and ψCal mRNAs are functional and encode different polypeptide products. These data are compatible with the proposed model that alternative RNA splicing of the transcript(s) of the calcitonin gene ultimately results in the production of different polypeptide products.**

Although transcriptional regulation of gene expression is clearly a critical determinant of mRNA production, the existence of intervening sequences in DNA raises the possibility of regulation at the level of mRNA splicing (1, 2). The potential versatility provided by generating multiple mRNAs via selective splicing patterns has been elegantly exploited in several viral systems, and evidence for such alternative splicing events has been suggested for several cellular transcripts (3–9). We have suggested that such selective RNA processing events may be important determinants of the expression of the gene encoding the polypeptide hormone calcitonin (8). Gilbert (2) predicted that introns may separate functional domains of the encoded proteins. We propose that functional domains in genes of the endocrine system can represent alternate polypeptide hormones which may be differentially expressed based upon alternative RNA splicing events.

A series of molecular events reproducibly accompany the apparently spontaneous and permanent switching, in serially transplanted rat medullary thyroid carcinoma lines, to calcitonin production states 1/30th to 1/10th the levels of the parental tumor (8). These molecular events unexpectedly include continued transcription of the calcitonin gene to an extent comparable to that observed prior to switching and a marked increase in steady-state levels of specific putative calcitonin mRNA precursors. We have suggested that the consequence of these unexpected changes is the reduction of calcitonin mRNA levels and a corresponding appearance of one or more alternative mRNAs, possibly encoding new protein products (8). Thus, reduction of calcitonin biosynthesis is associated with production of a new RNA retaining partial structural identity to mature calcitonin mRNA. Based upon this evidence, we have proposed that a splicing choice mechanism could be responsible for generating multiple mRNAs (8).

In order to document further that RNA processing events are responsible for the switching phenomena, we have generated plasmids containing cDNA inserts specific for the parental and variant RNAs. We establish in this report that the information that encodes both RNAs is present in the isolated, cloned, calcitonin gene. We further establish the presence of both calcitonin and variant mRNA sequences in common nuclear RNA transcripts in both switched and nonswitched cells. This suggests that the formation of alternate mRNAs occurs by selective RNA splicing events. The functional identification of two distinct RNA species was confirmed by *in vitro* translation and hybrid-arrest-of-translation analyses, establishing that each RNA species encodes a different protein. We further suggest that the production of these mRNAs provides an accurate stage-specific marker of calcitonin tumor differentiation. Identification of the variant RNA in normal thyroid cells suggests the physiological relevance of these switching mechanisms. These observations support the previous suggestion (8) that a single endocrine gene encodes more than one protein product.

MATERIALS AND METHODS

Cell-Free Protein Synthesis. RNA extractions and oligo(dT) chromatography were performed using frozen rat medullary thyroid carcinoma tissue as described (10). The extracted RNA was used to direct cell-free protein synthesis in a wheat embryo cell-free protein synthesizing system, as described (11). Radiolabeled protein products were fractionated by the $NaDodSO_4$/polyacrylamide gel electrophoresis method of Laemmli (12).

Hybridization-Arrested Translation. Rat calcitonin cDNA clone pCal was prepared, selected, and subjected to DNA sequence analysis as described (10). Total structural analysis of this cDNA clone (unpublished data) revealed that it contains the entire 411-nucleotide coding region, and 68 and 148 nucleotides of 5′ and 3′ noncoding sequence information, respectively. Linear, double-stranded plasmid DNA (1–4 μg), purified by rate zonal sedimentation and linearized by using *Eco*RI, and poly(A) (20–80 ng), were taken up in 2 μl of H_2O and heated at 100°C for 1 min in sterile siliconized 1.5-ml Microfuge tubes prior to use in hybridization-arrested translation (13). The samples were then brought to a final volume of 25 μl with 10 mM Pipes, pH 6.4/0.4 M NaCl/80% deionized formamide and transferred to a 65°C water bath. Poly(A)-selected RNA was dissolved in H_2O to a final concentration of 0.5 μg/μl and boiled 30 sec, and a 2-μl aliquot was added to each hybridization. The incubation

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Abbreviations: ψCal, pseudo-calcitonin; kb, kilobase(s).

temperature was allowed to fall slowly (<1°C/5 min) from 65°C to 42°C. After the hybridization, 200 μl of H_2O containing 10 μg of wheat germ tRNA was added to each reaction mixture. Half of each reaction mixture was boiled for 2 min and quick-frozen in a dry ice/acetone bath. Both the hybrid and the boiled/melted samples were made 0.35 M in NaCl and precipitated overnight at −20°C with 2.5 vol of 95% ethanol. Pellets were reprecipitated from 200 mM NaCl and ethanol and washed twice with 200 μl of cold 95% ethanol. Pellets were lyophilized and resuspended in 2.5 μl of [^{35}S]methionine (12.5 μCi; 500–1000 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) and translated in a wheat germ translation system (10).

Blot Analysis. Total poly(A)-rich RNA was denatured by reaction with glyoxal (14) and subjected to electrophoresis on 1.5% agarose slab gels buffered with 50 mM Tris·HCl/20 mM sodium acetate/1 mM EDTA, pH 7.9. After electrophoresis the gel was gently shaken for 20 min in 50 mM NaOH in the presence of acridine orange, photographed, and neutralized with two washes of 0.2 M sodium acetate (pH 4.3). The RNA was then transferred to diazotized paper, and prehybridization washes were performed as described (8). ^{32}P-Labeled probe was generated by nick-translation of the purified plasmid to a specific activity of $1–2 \times 10^8$ cpm/μg (15). Labeled plasmid ($0.5–5 \times 10^6$ cpm/ml) was hybridized to the blot for 12–14 hr, which then was washed and radioautographed.

cDNA Synthesis and Cloning Procedures. Size-fractionated poly(A)-rich RNA 800–1500 nucleotides long was used as initial substrate to generate double-stranded DNA for insertion into the *Pst* 1 site of pBR322 and transformation into *Escherichia coli* K-12 (SF8) (10). Two thousand colonies identified by differential antibiotic sensitivity as including insert-containing plasmid were selected for further characterization.

The cDNA probes were prepared by reverse transcription of size-fractionated poly(A)-rich RNAs from appropriate tissues; oligo(dT) was used as primer (16). The probes were used in the *in situ* colony hybridization assay of Grunstein and Hogness (17). Plasmids from selected colonies were amplified by using chloroamphenicol at 125 μg/ml and purified by the cleared lysate technique of Clewell and Helinski (18). DNA sequence analyses were performed by the method of Maxam and Gilbert (19).

Molecular Cloning of the Calcitonin Gene and Blot Analysis. A library of partial *Eco*RI digests of total rat liver nuclear genomic cDNA cloned into a λCh4A vector was generously provided by T. Sargeant (20). The library was screened by the plaque hybridization assay of Benton and Davis (21), and 6 positive plaques were identified from more than 1,000,000 plaques screened. Six clones were plaque purified until homogeneous, and phage DNA was prepared and analyzed (unpublished data). The identity of the clone as the calcitonin genome has been established by the precise correspondence of the genomic DNA sequence to cloned calcitonin cDNA (unpublished data). Calcitonin genomic DNA from this phage preparation and from total rat genomic DNA were utilized for Southern blot analysis (22).

RESULTS

Cloning of Pseudo-Calcitonin (ψCal) mRNA-Specific cDNA Sequences. Previous results with a calcitonin cDNA clone used as a hybridization probe demonstrated that variation in the expression of the calcitonin gene may be associated with the production of a distinct calcitonin-like mRNA (8). The synthesis of this new RNA occurs in tumors that switch from "high" to "low" levels of calcitonin production. Although this RNA hybridizes with the calcitonin in cDNA clone pCal, its larger size (1250–1350 vs. 1050 nucleotides) suggests that it contains sequences not present in mature calcitonin mRNA. To characterize this calcitonin-like or ψCal mRNA and its mechanism of formation further, a plasmid containing DNA complementary to the sequence unique to the new RNA was isolated. The strategy used a recombinant cDNA library prepared from 10–12S poly(A)-rich RNA isolated from rat medullary thyroid carcinoma tissue (8). Twin replica filters of 1920 colonies from this library were screened in a "plus/minus" type assay using ^{32}P-labeled cDNA enriched for calcitonin ("high" cDNA) or ψCal sequences ("low" cDNA) as probe. Plasmids giving a positive signal with the ψCal probe and a negative signal with the mature calcitonin probe were examined in more detail.

These isolates all hybridized to RNA from low-calcitonin-producing tumors but not to RNA from high-calcitonin-producing tumors. One plasmid containing an insert >500 base pairs long was subjected to complete DNA sequence analysis and found to share no region sequence with the calcitonin cDNA clone. The DNA sequence is not presented because, in the absence of a 5′ initiation codon in the clone, the identification of the correct reading frame is ambiguous and, therefore, the predicted polypeptides cannot yet be stated with absolute confidence. However, the large size of the insert suggests that ψCal cannot simply be formed by a 150- to 250-nucleotide extension of calcitonin mRNA. The clone to this mRNA is subsequently referred to as pψCal.

Nuclear Transcripts Contain Calcitonin and ψCal Sequences. Based on analysis of nuclear RNA profile in high- and low-calcitonin-producing tumors, we suggested that a splicing-choice mechanism may play a role in generating the 1050-nucleotide calcitonin mRNA or the 1250-nucleotide, ψCal mRNA (8). This model requires that both calcitonin and ψCal sequences be simultaneously present on one or more nuclear transcripts. To test this prediction, RNA from high- and low-calcitonin-producing tumors was size-fractionated and analyzed by RNA blotting procedures with pCal or pψCal as probe. Hybridization with pCal supports our early findings demonstrating that switching from high calcitonin production is associated with an increased abundance of several nuclear transcripts and the appearance of ψCal mRNA (Fig. 1). When RNA from high-producer cells was challenged with the low-specific clone (pψCal), no hybridization to mature 1050-nucleotide calcitonin mRNA was observed. This failure to hybridize was expected because this clone contains no sequence information present in calcitonin mRNA. Unexpectedly, pψCal did hybridize to an 1150-nucleotide RNA present in low levels in high-producer tumor RNA. When RNA from low producers was challenged with pψCal probe, the same precursors that had been previously identified by hybridization to pCal were visualized. Thus, the same nuclear transcripts apparently contain sequences specific to both calcitonin and ψCal mRNAs. This is consistent with the hypothesis that these two mRNAs are derived from a common precursor.

Both Cal and ψCal mRNAs Are Derived from the Same Gene. The demonstration that both calcitonin and ψCal sequences are present on the same RNA suggests that they could be transcribed from the same gene but does not exclude the existence of several related genes. To determine the genomic origins of Cal and ψCal mRNAs, we first determined whether there are single or multiple calcitonin genes. Hybridization of nick-translated pCal to Southern blots of DNA from rat liver or calcitonin-producing medullary thyroid carcinoma tumors showed a pattern of bands expected from a single gene (Fig. 2*A*); one major *Eco*RI fragment [8.2 kilobases (kb)] was identified. In addition, a faintly hybridizing band (6.3 kb) was occasionally observed in the *Eco*RI digests. This hybridization was so slight

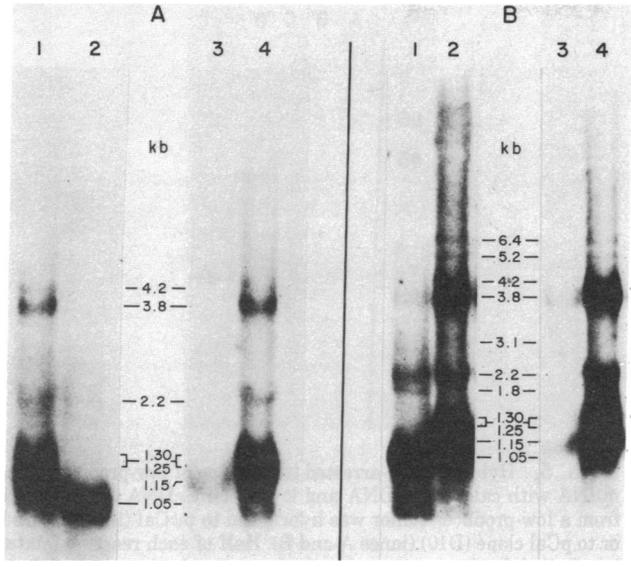

FIG. 1. Analysis of calcitonin-reactive RNA species with two different cDNA clones. Total poly(A)-rich RNAs prepared from low- or high-producer tumors were denatured, size fractionated, transferred to diazotized paper, and hybridized against nick-translated cDNA clones. (*A*) pCal was hybridized against 10 μg of poly(A)-rich RNA from low-producer tumors (lane 1) or high-producer tumors (lane 2). After elution of hybrid, the low-specific cDNA clone pψCal was hybridized against the same RNA blot from high-producer tumors (lane 3) and from low-producer tumors (lane 4). (*B*) Similar hybridizations. RNA from high-producer tumors was used in lanes 1 and 3 and from low-producer tumors, in lanes 2 and 4. Sizes are shown in kilobases (kb).

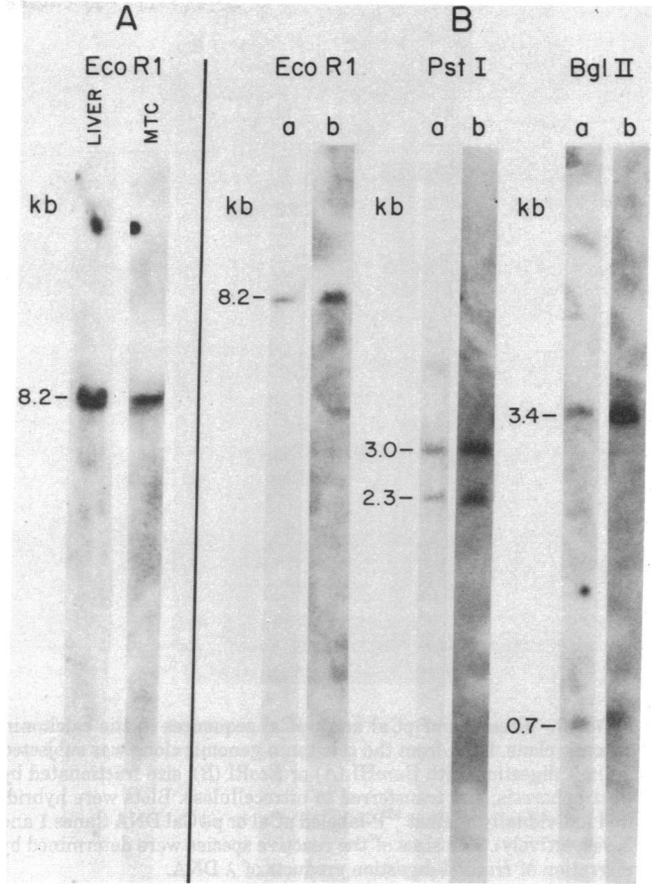

FIG. 2. Southern blot analysis of the calcitonin gene. (*A*) Total rat genomic DNA from liver (25 μg) or medullary thyroid carcinoma tissue (10 μg) was digested with *Eco*RI, fractionated by agarose gel electrophoresis, transferred to nitrocellulose, and hybridized against ^{32}P-labeled pCal. (*B*) Comparative hybridizations of high molecular weight liver DNA (lane a) and DNA from the genomic clone (lane b) after cleavage with the indicated enzymes. *Hin*dIII-digested λcI857 provided size (in kb) markers.

that it did not photograph well. The significance of these bands is unclear.

To determine whether pCal and pψCal sequences are linked, the reactive chromosomal region was isolated from a rat genomic library. Six independently isolated clones contained the same structural and flanking sequences. Southern blot analysis was used to compare the chromosomal organization to that of the genomic clone (Fig. 2*B*). The identity of the hybridization patterns indicates that the isolated genomic clone contains the calcitonin gene. According to the above model, if Cal and ψCal are present in the same mRNA precursors, then they must be chromosomally linked.

The putative linkage of Cal and ψCal mRNA sequences was determined by hybridizing labeled cDNA clones to a Southern blot of restriction fragments from the calcitonin genomic clone (G-Cal$_1$). Both pCal and pψCal hybridized to sequences present in G-Cal$_1$ (Fig. 3) as predicted, establishing a chromosomal linkage. Together, these data are compatible with the model in which pCal and pψCal contain sequences derived from distinct domains of a unique calcitonin gene.

ψCal mRNA Encodes a Unique Protein. The presence of ψCal mRNA in tumors that fail to synthesize calcitonin suggests that this alternative mRNA may encode a unique polypeptide. *In vitro* translation of mRNA from thyroid medullary carcinoma tissue was used to identify a protein product that may be associated with ψCal mRNA synthesis. Total poly(A)$^+$RNA from tumors before and after reduction of calcitonin synthesis was prepared and translated in a cell-free system from wheat germ embryos. The [^{35}S]methionine-labeled products were analyzed by electrophoresis through NaDodSO$_4$/polyacrylamide gels and fluorography. RNA from high-calcitonin-producing tumors encoded the previously described precalcitonin peptide (Fig. 4) with a M_r of approximately 17,500 (p17.5). RNA from tumors that had switched from high to low calcitonin production directed the synthesis of a new and prominent protein of M_r 16,000 (p16). The markedly greater abundance of the smaller protein in low-producing tumors is consistent with the increased synthesis of ψCal mRNA.

We used the procedure of hybridization-arrest of translation to determine if p16 represents the translation product of ψCal mRNA. Because ψCal mRNA contains sequences present in both pCal and pψCal, hybridization to either of these plasmids should inhibit translation of the presumptive ψCal protein. pψCal selectively inhibited translation of p16 (Fig. 5). Hybridization in the absence of added cDNA or with pBR322, or melting of the hybrids, abolished this hybridization-arrested translation of p16. Thus, ψCal mRNA encodes a unique M_r 16,000 polypeptide specific to medullary thyroid carcinoma tumors that have switched from high to low calcitonin production.

DISCUSSION

Many of the small polypeptide hormones can be found as components of larger protein precursors and are apparently generated by posttranslational processing events (23–39). The constraints selecting for this organization are not clear but have certain genetic implications. The genes-in-pieces structure of the eukaryotic genome suggests that some of the protein domains defining individual hormones might be represented by

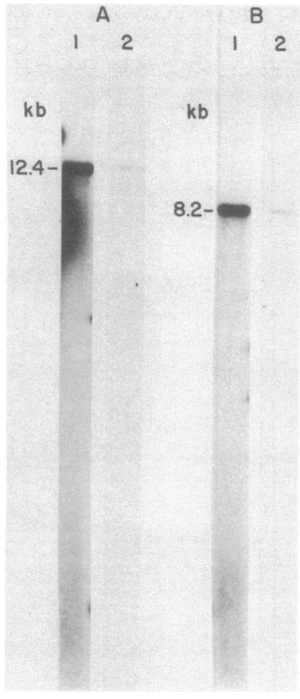

FIG. 3. Presence of pCal and pψCal sequences in the calcitonin genomic clone. DNA from the calcitonin genomic clone was subjected to limit digestion with *Bam*HI (*A*) or *Eco*RI (*B*), size fractionated by electrophoresis, and transferred to nitrocellulose. Blots were hybridized individually against ^{32}P-labeled pCal or pψCal DNA (lanes 1 and 2, respectively). The sizes of the reactive species were determined by migration of *Hin*dIII-digestion products of λ DNA.

comparable genetic regions separated from each other by intervening sequences. In such a model, an endocrine gene potentially could encode several polypeptide hormones whose ultimate production would be dependent upon posttranscriptional processing events to include the appropriate sequences ultimately expressed in the mature mRNA product. This model suggests that alternative posttranscriptional processing could

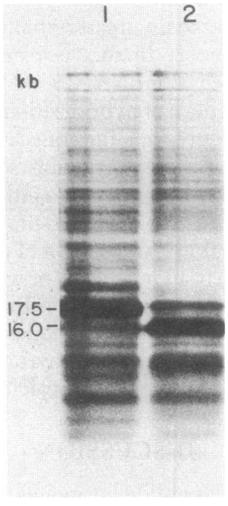

FIG. 4. Cell-free translation of mRNA from high- and low-producer tumors. The [^{35}S]methionine-labeled translation products directed by poly(A)-rich RNA from a high-producer tumor (lane 1) or a low-producer tumor (lane 2) were fractionated by NaDodSO$_4$/polyacrylamide gel electrophoresis. M_r is indicated $\times 10^{-3}$. Molecular size was determined by migration of known protein standards.

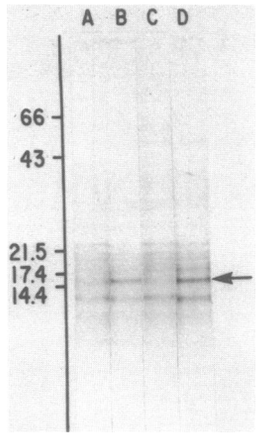

FIG. 5. Hybridization-arrested translation of low-producer tumor mRNA with calcitonin cDNA and low-specific cDNA clones. mRNA from a low-producer tumor was hybridized to pψCal (lanes C and D) or to pCal clone (D10) (lanes A and B). Half of each reaction mixture was boiled for 2 min, immediately frozen, and ethanol precipitated prior to translation (lanes B and D). The products of [^{35}S]methionine-labeled mRNA-directed protein synthesis were subjected to NaDodSO$_4$/polyacrylamide gel electrophoresis. No effects on translation were observed in the translation of either boiled or unboiled samples when hybridizations were performed with pBR322 without an insert, or in the absence of added RNA. The residual [^{35}S]methionine-labeled p16 present after translation is accounted for by a prominent M_r 16,000 wheat germ translation product. M_r standards are indicated $\times 10^{-3}$; arrow, migration of p16 translation product.

be used to generate mRNAs that insert additional domains or delete others.

We previously suggested (8), on the basis of an analysis of RNA precursors and mature transcripts, that differential RNA processing events may account for variation in the expression of the gene encoding the small polypeptide hormone calcitonin. In switching from a high to a low calcitonin-producing state, medullary thyroid carcinomas continue to transcribe large amounts of calcitonin-reactive nuclear RNA, and the apparent consequence of altered RNA splicing in these switched cells is the generation of a new cytoplasmic RNA distinct from calcitonin mRNA. Because of its structural homology, we call this product ψCal mRNA. In this study we further supported the model by cloning sequences uniquely present in the ψCal mRNA and using this clone as a probe to identify its synthetic pathway and genomic origins.

We have established that both calcitonin and ψCal sequences are present on a cloned genomic isolate of calcitonin DNA and can be identified on the same nuclear RNA transcripts. Because Southern blot analysis indicates that the calcitonin gene is apparently unique, these data suggest the calcitonin and ψCal sequences are encoded by the same chromosomal locus. Finally, evidence is presented to document that ψCal mRNA encodes a unique M_r 16,000 protein.

The data strongly favor a model in which two structurally different mRNAs encoding different protein products arise as a consequence of differential RNA processing events. The analysis cannot distinguish whether both mRNAs are generated from an identical primary transcript (in which case the observed polymorphism could be a result of *bona fide* splicing regulation) or the alternative splicing may itself be a consequence of other structural changes such as alternate 5′ or 3′ termini generated by differential transcription initiation or polyadenylylation. Variation in the structure of liver and salivary gland α-amylase has been suggested to be a consequence of the use of alternative transcription start sites (40, 41). Although this leads to structural

polymorphism, the extension is at the 5′ end of the mRNA and does not appear to alter the coding sequence; it may have other consequences. Analysis of the expression of genes of the immune system that encode heavy chains has suggested that its biological demands are also met in part by differential post-transcriptional events. The generation of alternative heavy chain immunoglobulin mRNA by differential poly(A) selection can have two described consequences. The first, involved in generation of μ_s or μ_m, results in a 3′ extension but conserves the major portion of the RNA (42, 43). The second is the simultaneous synthesis of μ and δ chains in cells in which RNA processing events are proposed to link a particular variable region to one of two constant-region domains (9). The similarity of these molecular events in gene expression in both the endocrine and immune systems suggests that they may be prototypic for other eukaryotic genes.

The generation of two mRNAs encoding separate protein products from the calcitonin gene implies the possibility that alternative hormones may be produced by ψCal mRNA. In fact, these observations are compatible with the idea that, in the endocrine system, the function of switching is to generate new polypeptide products. This report raises the possibility that a regulatory mechanism exists for generating additional hormones from a single chromosomal locus by posttranscriptional control mechanisms.

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